EXHIBIT B

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Increase in Plasma Homocysteine Associated with Parallel Increases in Plasma *S*-Adenosylhomocysteine and Lymphocyte DNA Hypomethylation*

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S-Adenosylmethionine and S-adenosylhomocysteine (SAH), as the substrate and product of essential cellular methyltransferase reactions, are important metabolic indicators of cellular methylation status. Chronic elevation of SAH, secondary to the homocysteine-mediated reversal of the SAH hydrolase reaction, reduces methylation of DNA, RNA, proteins, and phospholipids. High affinity binding of SAH to the active site of cellular methyltransferases results in product inhibition of the enzyme. Using a sensitive new high pressure liquid chromatography method with coulometric electrochemical detection, plasma SAH levels in healthy young women were found to increase linearly with mild elevation in homocysteine levels ($r = 0.73$; $p < 0.001$); however, S-adenosylmethionine levels were not affected. Plasma SAH levels were positively correlated with intracellular lymphocyte SAH levels ($r = 0.81$; $p < 0.001$) and also with lymphocyte DNA hypomethylation ($r = 0.74$, $p < 0.001$). These results suggest that chronic elevation in plasma homocysteine levels, such as those associated with nutritional deficiencies or genetic polymorphisms in the folate pathway, may have an indirect and negative effect on cellular methylation reactions through a concomitant increase in intracellular SAH levels.

An elevation in plasma homocysteine is a sensitive but nonspecific biomarker for an imbalance in the integrated pathways of one-carbon metabolism (1, 2). Chronic nutritional deficiencies in folate, choline, methionine, vitamin B_6, and/or vitamin B_{12} can perturb the complex regulatory network that maintains normal one-carbon metabolism and homocysteine homeostasis (3–7). Genetic polymorphisms in these pathways can act synergistically with nutritional deficiencies to accelerate the metabolic pathology associated with chronic disease states (8). Although several hypotheses have been proposed to explain the association between hyperhomocysteinemia and the thrombotic/atherosclerotic process occurring with occlusive cardiovascular disease, as yet none has been definitive (9–12). Similarly, increases in plasma homocysteine concentrations have been associated with increased risk of certain birth defects (13–16), but the underlying mechanism remains elusive. A major unanswered question is whether direct cellular toxicity of homocysteine is causally involved in pathogenesis or whether homocysteinemia is simply a passive and indirect indicator of a more complex mechanism.

Homocysteine is derived solely from methionine metabolism and is significantly recycled to conserve sufficient methionine for protein and *S*-adenosylmethionine synthesis. The interactive and interdependent pathways of the methionine/homocysteine cycle are diagrammed in Fig. 1 to emphasize the indirect effects of pathway perturbations on cellular methyltransferase reactions. The metabolic generation of homocysteine from methionine is initiated by the ATP-dependent transfer of adenosine to methionine via methionine adenosyltransferase. The product, *S*-adenosylmethionine (SAM),[1] is a priority for one-carbon metabolism because it is the methyl donor for most cellular methyltransferase reactions. In addition to DNA methylation, SAM-dependent methyltransferase activity is essential for hundreds of other cellular methylation reactions including synthesis of creatine in the liver, membrane phosphatidylcholine synthesis, central nervous system neurotransmitter synthesis, methylation/detoxification, and RNA and protein methylation (17). After transfer of the methyl group, SAM is converted to *S*-adenosylhomocysteine (SAH) within the active site of the methyltransferase enzyme. Because most methyltransferases bind SAH with higher affinity than SAM, they are subject to potent product inhibition by SAH (18). Thus, the efficiency of methyltransferase reactions is absolutely dependent on efficient product removal of SAH. This is effectively accomplished by SAH hydrolase (SAHH), an enzyme that appears to act in close proximity to the methyltransferases, at least in the nucleus (19). The crystal structure of SAHH has been recently reported, and interestingly, the polypeptide folding pattern at the catalytic domain of SAHH is almost identical to that reported for the DNA methyltransferases and suggests that SAH molecules can travel easily between the catalytic pockets of the two enzymes (20). This binding site similarity further supports an important role for excess SAH in the regulation of methyltransferase activity (20).

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[1] The abbreviations used are: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; SAHH, SAH hydrolase; HPLC, high performance liquid chromatography; tHcy, total homocysteine; THF, tetrahydrofolate; DMG, dimethylglycine.

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transfected CMV-GFP DNA (Fig. 2, *B* and *E*). Because methylation inhibits the expression of CMV-GFP (30), inhibition of demethylation of CMV-GFP results in reduction of GFP protein expression (Fig. 3, *A* and *C*), illustrating that AdoMet affects both demethylation of DNA and gene expression. This association of inhibition of demethylation and silencing of gene expression prompted us to rule out the possibility that AdoMet has a general, methylation-independent inhibitory effect on gene expression, or a general toxic effect, which also might result in inhibition of expression.

It is possible that AdoMet increases histone methyltransferase activity, resulting in hypermethylation of Lys-9 on H3 histones, which has been shown to correlate with inhibition of acetylation (40, 41). Inhibition of acetylation was shown to inhibit expression and demethylation of CMV-GFP (31). To address this alternative possibility, we measured in parallel the effects that AdoMet might have on methylated as well as unmethylated CMV-GFP plasmid, both transfected and treated with exogenous AdoMet under equivalent conditions. We first show that AdoMet treatment does not result in *de novo* methylation of unmethylated CMV-GFP (Fig. 2*E*). Thus, exogenous AdoMet does not stimulate DNA methylation as might be predicted by the current hypothesis of the mechanism of action of AdoMet. Second, we show that exogenous AdoMet does not inhibit expression of unmethylated CMV-GFP under conditions where a clear inhibition of expression of methylated CMV-GFP is observed (Fig. 3, *B* and *D*). Thus AdoMet specifically affects the expression of methylated genes. To our knowledge, this is the first demonstration that AdoMet specifically targets methylated DNA. This result also rules out the possibility that AdoMet exerts a general toxic effect on the cell. Our data therefore demonstrate that exogenous AdoMet specifically affects methylated DNA and prevents its expression. This most probably occurs by inhibiting an endogenous demethylase activity, resulting in hypermethylation of CMV-GFP and methylation-dependent repression.

We used the product of AdoMet-dependent methyltransferase reactions, AdoHcy, as a control. AdoHcy differs from AdoMet by a single methyl group. We show that AdoHcy has no effect on either gene expression (Fig. 3, *A* and *C*) or demethylation (Fig. 2, *B* and *E*). Taken together, these results indicate that both activities of AdoMet, inhibition of demethylation and inhibition of gene expression, are tightly associated and that they are both dependent on the methyl moiety in AdoMet.

In addition, we show that AdoMet directly inhibits recombinant MBD2/dMTase as well as demethylase activity extracted from HEK 293 cells (Fig. 4) in a dose-dependent manner using an *in vitro* assay (Fig. 5). AdoHcy does not inhibit MBD2/dMTase at the same concentrations (Fig. 5). Because an increase in intracellular AdoHcy was previously shown to be associated with hypomethylation (11), we tested the possibilities that AdoHcy (*a*) stimulates MBD2/dMTase activity, and (*b*) competes with AdoMet binding to MBD2/dMTase and relieves AdoMet inhibition. Our results suggest that AdoHcy does not interact with MBD2/dMTase and that it has no effect on AdoMet inhibition of this enzyme *in vitro*. Our results support the conclusion that the methyl group in AdoMet is required for its interaction with MBD2/dMTase. Although our results demonstrate that exogenous AdoMet inhibits demethylase activity *in vitro* and in living cells, there is no evidence that the intact AdoMet is the inhibitor. Because AdoMet is not intrinsically stable, particularly at physiological pH, it is difficult to assess whether AdoMet or a breakdown product is the inhibitory compound. At the AdoMet concentrations (mM) used in our studies, micromolar or even nanomolar concentrations of breakdown products of AdoMet may be present. Further experiments are required to test this possibility. Nevertheless, our experiments demonstrate that pharmacological administration of AdoMet inhibits active demethylation and alters gene expression.

Further experiments are also required to determine whether MBD2/dMTase is responsible for demethylation of our methylated plasmid in HEK 293 cells. Nevertheless, the fact that AdoMet inhibits both recombinant MBD2/dMTase and endogenous demethylase activities (Fig. 5) provides support for the hypothesis that demethylase(s) is inhibited by AdoMet. Thus, in addition to its role as a cofactor of transmethylation reactions, AdoMet can also act as a regulator of DNA methylation metabolism by inhibiting demethylase activity.

Our data further emphasize that the demethylase side of the methylation equilibrium has to be taken into account when dissecting the mechanism of action of drugs that modify the DNA methylation pattern. Based on our data, we suggest that AdoMet can alter DNA methylation patterns by inhibiting demethylase, which is expressed in some or most cells (Fig. 6). In this case, a reduction in the intracellular levels of AdoMet by methyl-deficient diets removes this inhibition, and increases the demethylase tone, resulting in active demethylation of DNA that could take place even in postmitotic tissue. Interestingly, AdoMet has recently been shown to inhibit the overall demethylation of a CG site in the 5′ of the myogenin gene during C2C12 differentiation (21). However, this report did not determine whether AdoMet stimulated DNMT or inhibited DNA demethylase.

What are the potential implications of the inhibition of demethylase by AdoMet? It is well documented that a correlation exists between reduced intracellular AdoMet (either as a consequence of decreased folate intake or pharmacological intervention) and an increase in cell proliferation and tumorigenesis (6, 35, 36, 38). In addition, other studies have shown that a decrease in dietary folate, or a depletion of intracellular AdoMet, results in DNA hypomethylation (6, 12, 36, 37). This is consistent with the well documented observations of global hypomethylation in cancer cells (42). There is evidence that the tumor protective mechanism of AdoMet involves DNA methylation because this protection is removed when the animals are co-treated with 5-azacytidine and AdoMet (19). In accordance with this hypothesis, we have recently shown that antisense inhibition of MBD2/demethylase inhibits tumorigenesis (43). It is tempting to speculate that certain genes that are required for anchorage independent growth might be inhibited by methylation and activated by demethylase activity. Inhibition of the demethylase tone by AdoMet is proposed to result in silencing of these genes. If the mechanism of action of AdoMet in inhibiting tumorigenesis involves inhibition of demethylation, it would support the hypothesis that demethylation plays a causal role in tumorigenesis, and serve as a warning against using inhibitors of DNA methylation as anticancer agents.

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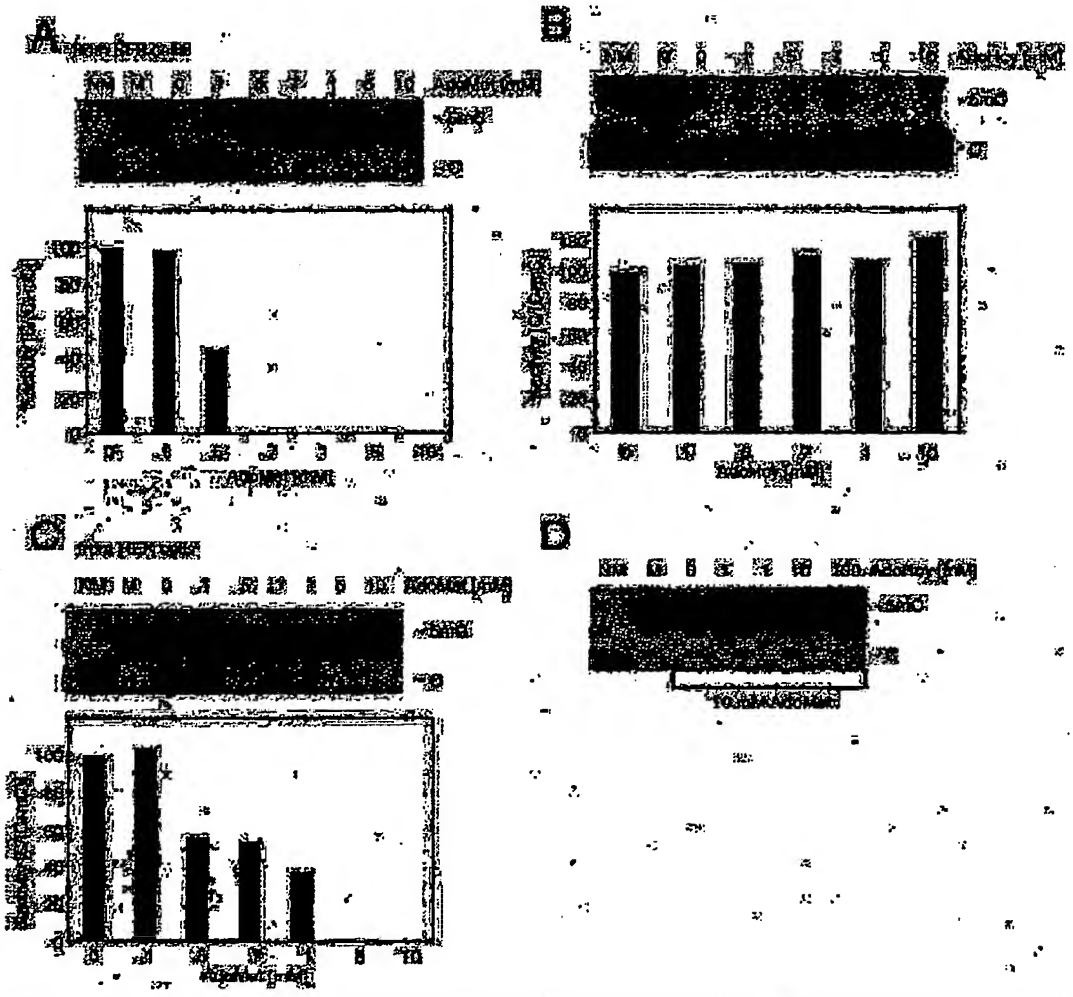

FIG. 5. **AdoMet, but not AdoHcy, inhibits demethylation activity *in vitro*.** *A* and *C*, ^{32}P-prelabeled methylated DNA from *M. leisodeikticus* was incubated with either MBD2/dMTase (*A*) or HEK 293 cells' extracted demethylase (*C*) and increasing concentrations of AdoMet. The autoradiography and quantification of one representative TLC plate is shown. Percent activity was calculated as described under "Materials and Methods." *B*, increasing concentrations of AdoHcy were added to the demethylase reaction; a representative experiment is shown. *D*, increasing concentrations of AdoHcy were added to reaction mixtures containing 10 mM AdoMet and demethylase activity was determined. *NM*, unmethylated control; *M*, methylated control; *5mC*, 5-methyldeoxycytidine 3'-monophosphate; *C*, deoxycytidine 3'-monophosphate.

inhibitor as was demonstrated for methylene tetrahydrofolate reductase (39). Furthermore, we do not know how MBD2/dMTase recognizes AdoMet on a structural basis.

DISCUSSION

The currently accepted mechanism for the effects of the methyl donor AdoMet on DNA methylation and tumorigenesis is founded on the assumption that the DNA methylation reaction is irreversible and defined exclusively by the DNMT. Taking advantage of our previously developed assay of demethylase activity in living cells (Fig. 1), we tested an alternative hypothesis: that AdoMet inhibits demethylase activity. If the steady state methylation status of DNA is maintained by an equilibrium of DNMT and demethylase activities (29), then inhibition of the demethylase side of the equilibrium should result in hypermethylation. Therefore, the reported DNA hypermethylation effects of exogenous AdoMet might be caused in part by inhibiting the level of demethylase activity in tumor cells. The main advantage of the system used in this paper is that it studies active demethylation exclusively, without interference from either replication-dependent passive demethylation or *de novo* DNMT activities (30).

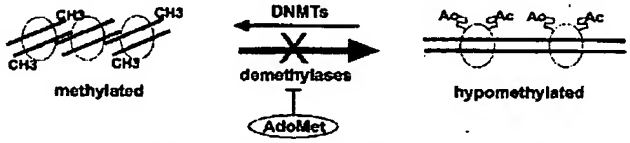

FIG. 6. **Possible model depicting how AdoMet may alter DNA methylation patterns and exert a chemoprotective effect.** The steady state methylation pattern of a gene is determined by an equilibrium of DNMTs and DNA demethylases acting upon it. In cells where DNA demethylase is overexpressed, certain genes may have a tendency to become hypomethylated, and some of these genes may promote anchorage independent growth and tumorigenesis. In this case, the administration of AdoMet would have a tumor protective effect by inhibiting demethylation and shifting the equilibrium to the normally methylated state.

We show here that exogenous AdoMet inhibits TSA-stimulated demethylation of ectopically methylated and transiently

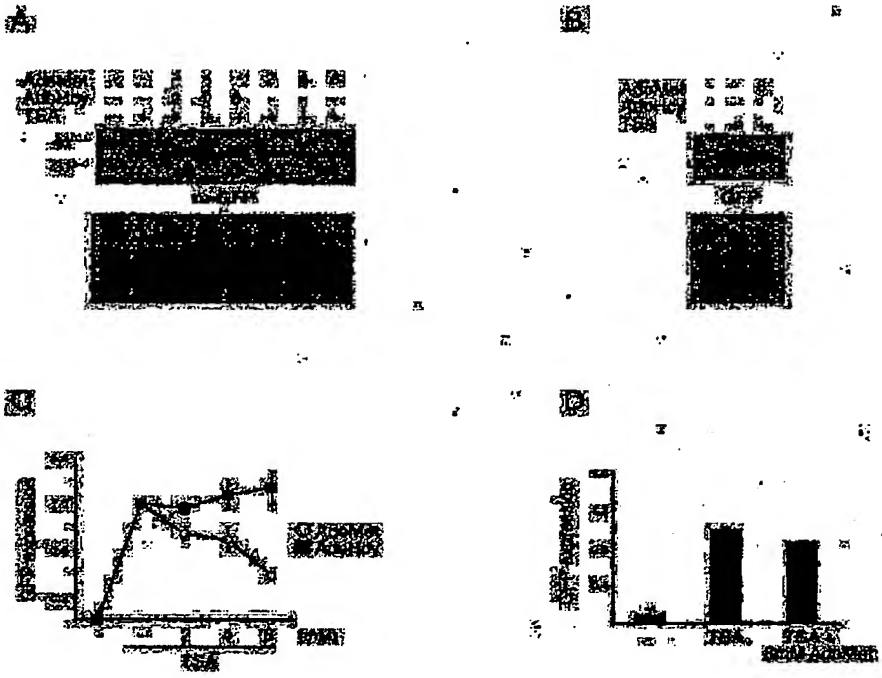

FIG. 3. **AdoMet reduces TSA-induced expression of methylated CMV-GFP.** *A* and *B*, HEK 293 cells were transiently transfected with either methylated CMV-GFP plasmid (m-GFP) (*A*) or unmethylated CMV-GFP (*GFP*) (*B*) and treated with 0.3 μM TSA and with the indicated concentrations (mM) of AdoMet or AdoHcy. Total cell extracts were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane, and GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334). Membranes were stained with Ponceau S to show equal loading. *C*, experiments such as those shown in *A* were performed in triplicate and quantified by densitometry. Results are presented as arbitrary units relative to the level of GFP expression in the presence of TSA alone that was normalized to one. ○, AdoMet; □, AdoHcy. *D*, quantification of triplicate experiments as shown in *B*; the averages ± S.D. are presented.

the value obtained for the demethylase reaction in the absence of inhibitor (0 mM AdoMet/AdoHcy). Conversion of methyl-dCMP to CMP was greatly reduced at 0.5 mM AdoMet and abolished completely at concentrations higher than 0.7 mM.

In contrast to AdoMet, no inhibition of demethylation occurred in the presence of increasing concentrations of AdoHcy (Fig. 5*B*). These results indicate that the small differences in the chemical structure (methyl group and positive charge on the sulfur) between AdoMet and AdoHcy are responsible for their different interactions with the MBD2/dMTase.

To test whether AdoMet inhibits endogenous HEK 293 demethylase activity, we extracted demethylase from HEK 293 cells using Q-Sepharose fractionation as previously described (27) and incubated it with increasing concentrations of AdoMet. The results shown in Fig. 5*C* indicate that, similar to recombinant MBD2/dMTase, the demethylase activity extracted from HEK 293 cells is inhibited by 50% at 0.5 mM AdoMet. Taken together, the above experiments demonstrate that AdoMet can inhibit the *in vitro* demethylation activity of recombinant MBD2/dMTase as well as endogenous demethylase extracted from HEK 293 cells.

AdoMet and AdoHcy compete for binding to the catalytic site on DNMTs. It was therefore proposed that the ratio of AdoHcy to AdoMet determines DNMT activity as discussed in the Introduction. AdoHcy inhibits DNMTs whereas increased AdoMet offsets this inhibition. We therefore determined whether a similar relationship applies to MBD2/dMTase. A competition experiment between AdoMet and AdoHcy is presented in Fig. 5*D*. Increasing concentrations of AdoHcy were added in the presence of an inhibitory concentration of AdoMet (10 mM), in a series of demethylation reactions. The results of this experiment illustrate that even a 10-fold concentration excess of AdoHcy to AdoMet does not diminish inhibition of the demethylase reaction by AdoMet. This is consistent with the hypothesis that AdoMet has a higher affinity for MBD2/dMTase as compared with AdoHcy. Further studies are necessary to elucidate the mode of inhibition: whether AdoMet is a competitive inhibitor with the substrate DNA or an allosteric

FIG. 4. **Partial purification of His-MBD2/dMTase from Sf9 cells.** *A*, nuclear extracts of Sf9 cells infected with MBD2/dMTase baculovirus were subjected to chromatography on Q-Sepharose and eluted with a stepwise gradient of NaCl. Fractions were assayed for demethylase activity using a ^{32}P-prelabeled methylated DNA from *M. leisodeikticus*. Demethylation activity elutes almost exclusively at 0.4 M NaCl with some activity present in the 0.2 M NaCl fraction (*A*, *left panel*). The 0.4 M NaCl fraction was concentrated 10-fold and re-assayed (*A*, *right panel*). Following demethylation, the DNA was digested to 3′-mononucleotides that were separated by thin layer chromatography. The DNA incubated with the whole cell extract (*EX*) and the flow-through (*FT*) could not be recovered for activity analysis, most likely because of nuclease activities in the fractions. The MBD2/dMTase tightly binds to Q-Sepharose as shown by the fact that the washes are free of demethylation activity. *B*, the fractions were analyzed by Western blot using anti-Xpress antibody (Invitrogen) to demonstrate the presence of His-MBD2/dMTase (indicated by the *arrow*). The presence of the protein correlates with its activity in the elution profile, with almost all MBD2/dMTase detected at 0.4 M NaCl. Lower molecular weight bands in the extract and the bindings might be because of partial degradation of the protein during purification. *NM*, unmethylated control; *M*, methylated control; *5mC*, 5-methyldeoxycytidine 3′-monophosphate; *C*, deoxycytidine 3′-monophosphate.

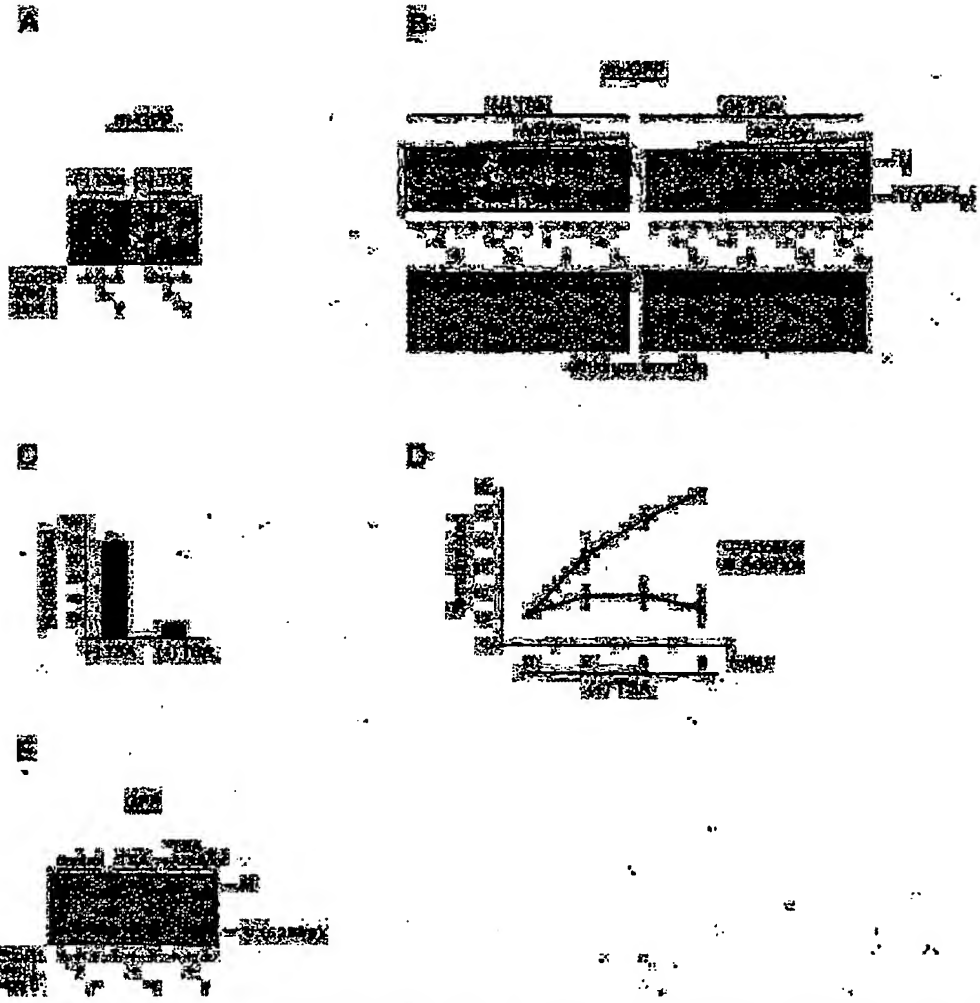

FIG. 2. **AdoMet inhibits active demethylation of CMV-GFP.** *A*, *B*, and *E*, either *in vitro* methylated CMV-GFP plasmid (*m-GFP*) (*A* and *B*), or unmethylated CMV-GFP (GFP) (*E*) were transiently transfected into HEK 293 cells. Cells were treated with a final concentration of 0.3 μM TSA (*+TSA*), or left untreated (*−TSA*), and increasing concentrations of either AdoMet or AdoHcy (2, 4, and 8 mM) were added. Cells were harvested 72 h post-transfection, and the methylation status of CMV-GFP was determined by *MspI/HpaII* restriction digestion and Southern blot analysis as outlined under "Materials and Methods" and in the legend to Fig. 1. *M*, methylated and *HpaII* undigested GFP; *U*, unmethylated and *HpaII* fully digested GFP (529 bp); *B*, *lower panel*, ethidium bromide-stained gels. *C*, the results of three independent experiments as shown in *A* were quantified by densitometry, and the average percent methylation remaining for each sample was calculated as outlined under "Materials and Methods" and charted ∓ S.D. *D*, the results of three independent experiments as shown in *B* were quantified as in *C* and the averages ± S.D. are presented. ○, AdoMet; □, AdoHcy.

vitro experiments should test whether AdoMet can act as an inhibitor of one or more demethylase activities.

First, His-tagged MBD2/dMTase was partially purified by chromatography on Q-Sepharose from cell extracts of Sf9 cells infected with the recombinant MBD2/dMTase construct as described under "Materials and Methods." Fractions were eluted with a stepwise gradient of NaCl and assayed for demethylation activity with a ^{32}P-prelabeled methylated DNA from *M. lysodeikticus* (Fig. 4, *A*, *left panel*). Conversion of methyl-dCMP to dCMP, whereas not extensive, was almost exclusively detected with the 0.4 M NaCl fraction (16% demethylation). This correlates with the peak presence of the His-tagged recombinant MBD2/dMTase protein in this fraction as demonstrated by Western blot analysis (Fig. 4*B*) using an anti-Xpress antibody. To confirm that the 0.4 M NaCl fraction contained demethylase activity, the fraction was concentrated 10-fold on a Microcon concentrator. As expected, the demethylase activity in the 0.4 M NaCl fraction increased accordingly (Fig. 4*A*, *right panel*); 5 μl of the concentrated fraction completely converted methyl-dCMP to dCMP for the same amount of DNA as used before (Fig. 4*A*, *left panel*).

Next, we determined whether AdoMet inhibits the demethylation activity of MBD2/dMTase. The aforementioned DNA was incubated with MBD2/dMTase from Sf9 cells in the presence of increasing AdoMet concentrations, and conversion of methyl-dCMP (mC) to dCMP (C) was assessed as above. Fig. 5*A* presents the autoradiography and quantification of one representative experiment. Because of the numerous steps involved in this assay, it is impossible to avoid small loading differences between samples. To determine percent activity, the percent demethylation, (C/(C + mC)), was thus calculated within each sample to control for these differences, and then normalized to

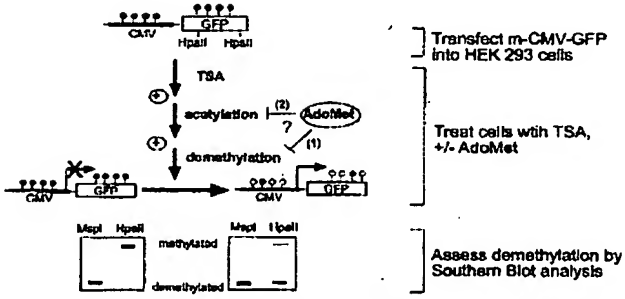

FIG. 1. **Outline of the model system used to assess the effects of AdoMet on active demethylation in living cells.** CMV-GFP plasmid is methylated *in vitro* and transiently transfected into HEK 293 cells. Histone acetylation is induced with TSA, which results in DNA demethylation by endogenous demethylase activity (30). The degree of demethylation is then measured by methylation-sensitive restriction digestion using *Hpa*II enzyme, which is followed by Southern blot analysis using a GFP-specific probe (*Aca*II-*Cfr*101 fragment). The possible mechanisms of action of AdoMet are indicated, either a direct inhibition of demethylase activity (1), or an indirect mechanism by first stimulating histone methylation and inhibiting histone acetylation, which would then inhibit active demethylation (2).

RESULTS

AdoMet Inhibits TSA-induced Active Demethylation of Ectopically Methylated and Transiently Transfected CMV-GFP in a Dose-dependent Manner—There have been several reports demonstrating that exogenous administration of AdoMet leads to DNA hypermethylation (19, 21, 35). Similarly, other studies have shown that a decrease in dietary folate, or a depletion of intracellular AdoMet, results in DNA hypomethylation (6, 12, 36, 37). However, it is not known whether the effects of AdoMet on methylation are because of changes in DNMT or DNA demethylase activities.

We utilized a previously described transient transfection-based assay system (Fig. 1 and Ref. 30) to study the effects of AdoMet on active demethylation of ectopically methylated DNA. In prior studies we have shown that the *in vitro* methylated CMV-GFP reporter plasmid is actively demethylated 72 h following transfection into HEK 293 cells when histone hyperacetylation is induced with TSA (30). Because CMV-GFP does not replicate nor is it *de novo* methylated in HEK 293 cells (30), this assay specifically measures active demethylation in a living cell.

We first determined the effects of increasing doses of AdoMet, or the product of AdoMet-dependent methyltransferase reactions, AdoHcy, on the demethylation of methylated CMV-GFP (Fig. 2). DNA was isolated from HEK 293 cells transfected with methylated CMV-GFP DNA and treated with either TSA and AdoMet, or TSA and AdoHcy. DNA was first linearized with the *Eco*RI restriction enzyme, followed by digestion with *Msp*I (which cleaves the sequence CCGG) or *Hpa*II (which cleaves the sequence CCGG only when it is not methylated). The demethylated *Hpa*II-digested 529-bp fragment (U) and the methylated *Hpa*II-undigested DNA (M) were quantified within the same lanes, and the percent methylation for CMV-GFP DNA in each lane was determined as [M/(U + M)] × 100. Values were then normalized to the total DNA per lane as determined by ethidium bromide staining. As can be seen in Fig. 2, *A* and *C*, the addition of TSA results in nearly complete demethylation of CMV-GFP by endogenous demethylase activity, as indicated by the complete *Hpa*II digestion of CMV-GFP to the 529-bp fragment (U). Upon the addition of increasing concentrations of AdoMet (Fig. 2, *B* and *D*), the percentage of methylated GFP remaining increases in a dose-dependent manner, illustrated by the decrease in the ratio of the 529-bp *Hpa*II fragment (U) to the undigested DNA (M). AdoHcy has an insignificant effect on the demethylation of CMV-GFP (Fig. 2, *B* and *D*), indicating that the methyl moiety of AdoMet is required for inhibition of demethylation.

We then determined whether AdoMet stimulates *de novo* methylation of an identical unmethylated CMV-GFP substrate. Fig. 2*E* illustrates that unmethylated CMV-GFP, transfected under identical conditions, does not get *de novo* methylated, even in the presence of 8 mM AdoMet. This indicates that AdoMet does not cause an increase in DNMT activity on ectopic CMV-GFP. Thus, the likely mechanism by which AdoMet causes hypermethylation of CMV-GFP in comparison with the TSA-treated control is by inhibiting its active demethylation by resident demethylases.

AdoMet Reduces TSA-induced Expression of Methylated CMV-GFP in a Dose-dependent Manner, but Has No Effect on Unmethylated CMV-GFP—A number of studies have shown that an increase in AdoMet inhibits gene expression (20, 21), however, it is not clear whether AdoMet specifically affects genes whose methylation state it alters exclusively, or whether it has a nonspecific effect on gene expression. We took advantage of the CMV-GFP system described above to address this question. We determined whether AdoMet influences the expression of either methylated CMV-GFP, whose methylation state is affected by AdoMet, or unmethylated CMV-GFP, whose methylation state is not affected by AdoMet. HEK 293 cells were transiently transfected and treated with TSA and either AdoMet or AdoHcy, as described in the previous section. Extracts were then prepared and subjected to a Western blot analysis using an antibody directed against GFP protein.

Fig. 3, *A* and *C*, illustrates that methylated CMV-GFP is completely repressed in untreated HEK 293 cells. This is as expected, because it is well documented that DNA methylation leads to gene silencing. The addition of TSA leads to a dramatic induction of GFP expression as expected from the complete demethylation following TSA treatment. Upon the addition of increasing amounts of AdoMet, GFP expression is decreased in a dose-dependent fashion. AdoHcy has no significant effect on the expression of methylated GFP, consistent with its lack of effect of DNA demethylation.

Because our system measures expression and demethylation that is dependent on histone hyperacetylation, there are two possible mechanisms whereby AdoMet exerts its effects on demethylation (Fig. 1). AdoMet could directly inhibit a demethylase activity, or it could inhibit histone acetylation, which we have previously shown leads to an inhibition of demethylation (30). If the latter were true, then AdoMet should also inhibit the TSA-induced expression of unmethylated GFP, whose expression is induced by histone acetylation as well. Fig. 3, *B* and *D*, indicates that this is not the case, because AdoMet has no significant effect on the induction of unmethylated GFP by TSA. The fact that AdoMet specifically affects the expression of a methylated copy of CMV-GFP, and not an unmethylated copy, supports the model that AdoMet inhibits gene expression by directly inhibiting the active demethylation of methylated CMV-GFP.

AdoMet but Not AdoHcy Inhibits Demethylation Activity in Vitro—To further confirm that the observed effect of AdoMet and AdoHcy is because of inhibition of active demethylation and not an indirect effect, *in vitro* studies with a recombinant MBD2/dMTase (the only demethylase characterized thus far) were performed (24). Because it is not certain whether MBD2/dMTase is responsible for the demethylation seen in HEK 293 cells, we also performed these studies with purified endogenous demethylase activity from HEK 293 cells. Together, these *in*

possibility that the *Hpa*II digestion was skewed by differences in loading, we used the ethidium bromide-stained gels as loading controls for the corresponding Southern blots. We normalized the values obtained upon the calculation [M/(U + M)] × 100 to the amount of DNA in each *Hpa*II lane as determined by quantification of the ethidium bromide-stained gels by NIH Image 1.62. The results of three independent experiments were quantified by densitometry (NIH Image 1.62).

AdoMet Preparations for in Vitro Studies

AdoMet was prepared as a 50 mM solution in distilled water by dissolving lyophilized powder (Sigma) in distilled water. AdoHcy was purchased from Sigma and dissolved in distilled water at a 50 mM concentration.

Purification of Recombinant MBD2/dMTase from Sf9 Cells

A fragment containing human MBD2/dMTase was excised from pCR2.1-dMTase (24) with *Bam*HI and *Xho*I and transferred to the Baculovirus expression transfer vector pBlueBacHis2 C (Invitrogen). PBlueBacHis2 C-MBD2/dMTase and Bac-N-Blue viral DNA were co-transfected into the Sf9 insect cell line, and recombinant viruses were isolated, identified, and amplified according to the manufacturer's protocol (Invitrogen) with no modifications. High titer P3 viral stocks were used for infections. Insect Sf9 cells were cultured in spinner flasks to a density of 2.5 × 10^6 cells/ml in Grace's insect cell culture medium supplemented (1×) from Invitrogen. For infection, 5 × 10^6 cells were plated in 10-cm tissue culture plates (Sarstedt) and allowed to settle and attach for 30 min. The culture medium was removed and was replaced with 10 ml of the same medium containing MBD2/dMTase virus at a multiplicity of infection of 10. The cells were cultured with the virus for 5 days at 27 °C and were then harvested by scraping in cold phosphate-buffered saline. Cell pellets from 10 plates were frozen and kept at −70 °C until they were used for enzyme purification. Frozen pellets were thawed in 5 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 500 mM NaCl, 0.05% Tween 20, 10% glycerol, and 10 mM imidazole) containing 1 μg/ml of the following protease inhibitors: aprotinin, leupeptin, and Pefablock. Protease inhibitors were added to all the solutions used in the purification. The homogenates were subjected to two cycles of freezing and thawing (5 min per step). DNA in the homogenate was sheered by passing through an 18.5-gauge needle 10 times. The extracts were then subjected to 15 cycles of sonication (10 s burst, 10 s gap per cycle at 20% of maximal output). The extracts were centrifuged at 10,000 × *g* for 35 min. The supernatant was transferred into a fresh tube and was recentrifuged for additional an 25 min at 15,000 × *g*. The extract was filtered through a 5-micron filter to remove any particulate matter and the buffer was exchanged on a PD-10 buffer exchange column (Amersham Biosciences) with buffer L (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) containing 50 mM NaCl. Recombinant MBD2/dMTase was partially purified by Q-Sepharose (Amersham Biosciences) ion exchange chromatography. Q-Sepharose beads (1 ml of swollen beads) were washed extensively and pre-equilibrated with buffer L containing 50 mM NaCl and divided into 3 equal aliquots. The cell extracts were sequentially bound three times to the 3 aliquots of Q-Sepharose beads in batch in 15-ml tubes by shaking gently on a Nutator for 45 min at 4 °C. Following each binding step, the bound beads and unbound supernatant were separated by centrifugation for 2 min at 1000 × *g* and the supernatant was transferred into a new tube and bound with new pre-equilibrated beads. The bound beads from the three binding steps were joined and resuspended in lysis buffer. The beads were washed in batchs 4 times with 5 ml of buffer L + 50 mM NaCl. For each washing step, the beads were incubated with the wash solution for 15 min and then separated from the wash supernatant by centrifugation for 2 min at 1000 × *g*. Following washing, the proteins were eluted in batchs (30 min per step) with a stepwise NaCl gradient in buffer L. Each elution step was analyzed for *in vitro* demethylase activity and for the presence of the recombinant His-tagged MBD2/dMTase by a Western blot analysis using the anti-Xpress antibody from Invitrogen as previously described. MBD2/dMTase peak elution is at the 0.4 M NaCl step. No demethylase activity was observed in the same fractions prepared in a similar manner from uninfected Sf9 cells. For concentration of the 0.4 M NaCl fraction, a Microcon YM-10 concentrator (Millipore) was used at 3300 × *g* and 4 °C. Spinning time varied according to the volume, and was 25 min for 500 μl.

Extraction of Endogenous Demethylase Activity from HEK 293 Cells

10 × 10-cm tissue culture plates of HEK 293 cells were used to prepare cell extracts as outlined in the previous section. Q-Sepharose fractionation was performed also as described above and as previously described (27).

Preparation of Substrate DNA for in Vitro Demethylation Assay

Methylation of Substrate—Typically, 25 μg of DNA from *Micrococcus lysodeikticus* (Sigma, Type XI, highly polymerized) were methylated with M.*Sss*I (60 units, New England Biolabs) and AdoMet (3.2 mM, New England Biolabs) in methylation buffer (NEbuffer2, New England Biolabs) in the presence of 50 mM EDTA for 3-4 h at 37 °C. Fresh AdoMet (3.2 mM) and enzyme (40 units) were then added before incubating at 37 °C for an additional 3–4 h. To achieve complete methylation, methylation was repeated after AdoHcy, which is a product of the methylation reaction and inhibits DNA methylation, and was removed using a Microcon 10 concentrator (Millipore) according to the manufacturer's protocol. The degree of methylation was verified by methylation sensitive restriction enzyme analysis (*Msp*I-*Hpa*II digestion) on aliquots of the reaction mixture. The DNA was purified by phenol-chloroform extraction (one part of either phenol or chloroform per three parts reaction mixture). Unincorporated nucleotides were removed by a Nap5 gel filtration chromatography (Amersham Biosciences) column. The Nap5 column was equilibrated with demethylation buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.0). DNA containing fractions were combined, concentrated on a Microcon 10, and subjected to a second Nap5 desalting column. DNA containing fractions were again concentrated as described above.

[α-^{32}P]dGTP Labeling of DNA—We then prepared either methylated or unmethylated DNA that is ^{32}P-labeled at G, the 3′ neighbor of the methylated C, as previously described (33) with the following modifications. 5 μg of either methylated or unmethylated DNA in 35 μl of double distilled water were denatured and annealed to a hexanucleotide primer by boiling for 10 min in the presence of 3 μl of random hexanucleotide mixture (0.2 A_{260}) (Roche Diagnostics). The primed DNA was then subjected to template-directed extension with the Klenow fragment of DNA polymerase I in the presence of labeled [α-^{32}P]dGTP, either methyl-dCTP (for methylated DNA) or dCTP for unmethylated DNA, dTTP, and dATP. The 3′ phosphate of all the 5′ neighbors of G including either C or methyl-C is labeled by this procedure. The labeling was performed in polymerase buffer (50 mM NaCl, 6.6 mM Tris-HCl, 6.6 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.4) with Klenow fragment I (10 units, Roche Diagnostics), methyl-dCTP (1 mM), and dCTP (1 mM), respectively, dATP, dTTP (1 mM each), and [α-^{32}P]dGTP (50 μCi, PerkinElmer Life Sciences) for 3 h at 37 °C. The reaction mixture was extracted with phenol and chloroform (one part of each per three parts reaction mixture). Trichloroacetic acid precipitation (2 ml of 10% trichloroacetic acid, 20 μg of herring sperm DNA) of an aliquot showed typically 80–95% labeling efficiency (260,000–320,000 cpm/μl, total of 150 μl). The DNA was purified by eluting it twice from a Nap5 column and concentrating on a Microcon 10 as described above with the following variation: distilled water was used for prewashing and elution in the second column. The final concentration was 5 ng/μl and the specific activity was typically 8.0–8.8 × 10^6 cpm/μg.

In Vitro Demethylation Assay

A typical reaction mixture (50 μl) consisted of 25 ng of ^{32}P-prelabeled DNA (prepared as described above) incubated in demethylation buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.0) with either the purified MBD2/dMTase (5 μl, ~5 ng) or the purified demethylase activity from HEK 293 cells (5 μl) for 24 h at 37 °C in either the absence or presence of AdoMet and AdoHcy, respectively. The DNA was extracted from the enzyme by incubation in 2 volumes of DNA extraction buffer (10 mM Tris-HCl, 0.5 M NaCl, 1% SDS) containing 0.1 unit of proteinase K (Roche Diagnostics) at 50 °C for 2 h. Subsequent phenol-chloroform extraction (one part of either phenol or chloroform per three parts of reaction volume) in the presence of tRNA (50 μg) as a carrier and ethanol precipitation with salt and 95% ethanol resulted in almost quantitative recovery of the input DNA. The DNA pellets were resuspended in distilled water (8 μl) and digested with micrococcal nuclease to ^{32}P-labeled 3′ mononucleotides as described elsewhere (27, 33). The labeled mononucleotides were separated by thin layer chromatography and visualized by autoradiography on a phosphorimaging plate, and the levels of cytosine (C) and 5-methylcytosine (mC) were quantified by the MCID-M4 software (Imaging Research Inc.). The percent demethylation (C/(C + mC)) was calculated per each sample and then normalized to the value obtained for the demethylase reaction in the absence of inhibitor (0 mM AdoMet/AdoHcy).

AdoMet is below the K_m for the enzyme, this has not been demonstrated as of yet. It is, however, possible that the main mechanism by which elevating AdoMet levels increases DNMT activity is by competing with AdoHcy, an inhibitor of DNMT. Such an indirect mechanism of activation might be relevant even if the basal level of AdoMet is above the K_m. Second, even if exogenous administration of AdoMet increases the activity of DNMT, it is not clear whether the normal level of enzyme is limiting, or whether the specificity of DNA methylation patterns is determined by the molecular activity of DNMT. There is no evidence to suggest that specific sites remain unmethylated in vertebrate genomes simply for the reason that the molecular activity of DNMT is limiting. Third, methyl-deficient diets cause hypomethylation in the liver whose cells are mostly postmitotic and do not replicate (5). If the mechanism of this hypomethylation involves only inhibition of DNMT, it could take effect only in cells that actively synthesize DNA. Because measurable demethylation is seen with these diets, this could only occur if a significant fraction of liver cells proliferate during the treatment. Whereas an increase in proliferation in the liver is seen as a consequence of methyl-deficient diets, it is not clear whether proliferation precedes or follows global hypomethylation.

The current hypothesis on the mechanism of action of AdoMet is based on the assumption that DNA methylation is a unidirectional and irreversible reaction, which is catalyzed by DNMT exclusively. However, an increasing list of data supports the hypothesis that DNA methylation *in vivo* is also fashioned by demethylase activity (22). A DNA demethylase activity was partially purified from human lung carcinoma A549 cells (23) and the protein MBD2b was shown to bear demethylase activity (24). The demethylase activity of MBD2 was disputed by several groups (25, 26), but our recent data demonstrated that ectopic MBD2/dMTase causes DNA demethylation in a promoter-specific manner (27). We therefore proposed that DNA methylation is a reversible reaction, and that the steady state DNA methylation status of a gene reflects a balance of methylation and demethylation (28, 29). Thus, it is possible that AdoMet increases methylation by inhibiting demethylation.

It is impossible to determine whether hypermethylation of a gene *in vivo* following chronic drug treatment is caused by either an increase in DNMT activity, as proposed by the current model, or inhibition of demethylase. A model system is required to study either methylation or demethylation in isolation from the reverse activity. We have recently described such a model (Fig. 1). When an unmethylated CMV-GFP plasmid is transiently transfected into the human embryonal kidney cell line HEK 293, it remains unmethylated throughout the transient transfection period up to 96 h, demonstrating that DNMTs do not target extrachromosomal DNA under the conditions of our experiment (30). When an *in vitro* methylated CMV-GFP plasmid is transiently transfected into these cells, it generally remains methylated. However, when histone acetylation is induced by trichostatin A (TSA), the plasmid is fully and actively demethylated by an endogenous demethylase activity. Because the plasmid does not replicate during the time frame of the experiment, this assay measures only active demethylation (30). This system thus allows us to determine the impact that different factors might have on demethylase activity. We have recently used this assay to illustrate that a protein that inhibits histone acetylation inhibits active demethylation in living cells (31).

In this paper we took advantage of this assay to test the hypothesis that exogenous administration of AdoMet inhibits demethylase activity in living cells. Using an *in vitro* demethylase assay, we then tested whether AdoMet inhibits both recombinant MBD2/dMTase activity extracted from infected Sf9 insect cells, as well as endogenous demethylase activity from HEK 293 cells. Taken together, our results support a new alternative hypothesis for the mechanism of action of AdoMet as a DNA hypermethylating agent.

MATERIALS AND METHODS

In Vitro Methylation of Substrates

CMV-GFP (pEGFP-C1 from Clontech; GenBank™ accession number U55763) was methylated *in vitro* by incubating 10 μg of plasmid DNA with 12 units of *SssI* CpG methyltransferase (New England Biolabs) in the recommended buffer containing 800 μM AdoMet for 3 h at 37 °C. Twelve units of *SssI* and 0.16 μmol of AdoMet were then added and the reaction was further incubated for 3 additional hours. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation, and complete methylation was confirmed by observing full protection from *HpaII* digestion.

Cell Culture and Transient Transfections

Human embryonal kidney HEK 293 cells (ATCC CRL 1573) were plated at a density of 7.5×10^4/well in a 6-well dish and transiently transfected with 80 ng of CMV-GFP (methylated or mock methylated) using the calcium phosphate precipitation method as described previously (32). 0.3 μM TSA was added 24 h post-transfection. After an additional 24 h, cells were treated with or without various concentrations of AdoMet or AdoHcy (2–8 mM). Cells were harvested 72 h post-transfection. Each experiment was performed in triplicate, and experiments were performed several times using different cultures of HEK 293 cells.

Western Blot Analysis

Whole cell extracts were prepared using radioimmunoprecipitation assay buffer according to the Santa Cruz Biotechnology protocol, and protein concentrations were determined using the Bradford reagent (Bio-Rad). 2.5 μg of protein were resolved on a 12.5% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Amersham Biosciences). After blocking the nonspecific binding with 5% skim milk, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334) at 1:500 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000, and enhanced chemiluminescence detection kit (Amersham Biosciences). Membranes were stained with 0.2% Ponceau S (Sigma) to determine loading of total protein in each lane. Both the Western blots and Ponceau-stained membranes were quantified using NIH Image 1.62 software, and the GFP signal was normalized to the total protein (which varied only slightly) in each lane.

Southern Blot Analysis

DNA was extracted from HEK 293 cells using the DNeasy Tissue Kit (Qiagen). DNA was first digested with 50 units of *EcoRI*, followed by digestion with 20 units of either *HpaII* or *MspI* restriction enzymes. Samples were subjected to electrophoresis on a 1.5% agarose gel and then transferred to Hybond-N^+ membrane (Amersham Biosciences). Blots were probed with a ^{32}P-labeled CMV-GFP cDNA probe (*AvaII-Cfr*101 fragment) synthesized using a random priming labeling kit (Roche Diagnostics). Membranes were hybridized at 68 °C for 4–6 h in a buffer containing 0.5 M sodium phosphate, pH 6.8, 1 mM EDTA, 7% SDS, and 0.2 mg/ml herring sperm DNA. Following hybridization, the membranes were washed twice for 10 min in a 5% SDS, 0.04 M sodium phosphate, pH 6.8, 1 mM EDTA solution, and then four times for 10 min in the same solution containing 1% SDS. The demethylation assay measures the fraction of GFP molecules that were demethylated using *HpaII* restriction enzyme, which cleaves unmethylated CCGG but does not cleave methylated CCGG sequences. The methylated GFP DNA remains intact following *HpaII* digestion and is identical to the fragment obtained following *EcoRI* digestion (indicated by *M* in Fig. 2*B*), whereas the unmethylated GFP DNA is cleaved by *HpaII* resulting in a 0.5-kb fragment (indicated by *U* in Fig. 2*B*). We scanned each *HpaII* digested lane and measured the intensity of the total signal hybridizing with the GFP probe in the same *HpaII* lane (including the unmethylated *U* and methylated *M* fragments), this value is equal to 100% of GFP molecules in the lane. We then determined the intensity of the unmethylated signal per *HpaII* lane, and divided this value (*U*) by the total signal for GFP (U + M) in the same *HpaII* lane. To exclude the